Exhibit C

L72

The Journal of
UROLOGY ®

Official Journal of the American Urological Association
Founded In 1917 By Hugh Hampton Young

ORIGINAL

SYSTEMIC BASIC FIBROBLAST GROWTH FACTOR INDUCES FAVORABLE HISTOLOGICAL CHANGES IN THE CORPUS CAVERNOSUM OF HYPERCHOLESTEROLEMIC RABBITS

QUNSHENG DAI, ARI D. SILVERSTEIN, MARK G. DAVIES, PER-OTTO HAGEN, CRAIG F. DONATUCCI AND BRIAN H. ANNEX*

From the Division of Cardiology, Department of Medicine (QD, BHA) and Division of Urology (ADS, CFD) and Atherosclerosis Research Laboratory (P-OH), Department of Surgery, Durham Veterans Affairs and Duke University Medical Center, Durham, North Carolina, and Department of Vascular and Endovascular Surgery, University of Rochester (MGD), Rochester, New York

ABSTRACT

Purpose: Hypercholesterolemia causes erectile dysfunction that is associated with abnormalities in vascular smooth muscle and endothelial cells. We determined the effects of basic fibroblast growth factor (bFGF) on corporeal tissue in hypercholesterolemic rabbits.

Materials and Methods: A total of 16 New Zealand White rabbits were fed a 1% cholesterol diet for 6 weeks and were randomly divided into 3 groups. Group 1 (5 rabbits) received 2.5 μg recombinant bFGF intravenously once and again 3 weeks later. Group 2 (6 rabbits) received 2.5 μg bFGF intravenously once and placebo 3 weeks later. Group 3 (5 rabbits) received placebo intravenously each time. Rabbits were continuously fed a 1% cholesterol diet and sacrificed 3 weeks after the last treatment. Smooth muscle, endothelial cell and collagen content were assessed by immunohistochemistry and histochemical staining of corporeal tissue. Vascular endothelial growth factor (VEGF) protein and mRNA expression were assessed by enzyme-linked immunosorbent assay and reverse transcriptase-polymerase chain reaction.

Results: Corporeal smooth muscle content was greater in groups 1 and 2 (35.24% ± 4.25% and 24.79% ± 3.39%, p <0.01) vs group 3 (19.68% ± 2.94%, vs groups 1 and 2 p <0.001 and <0.05, respectively). Endothelial cell and collagen content were similar among the groups. VEGF protein was increased in group 1 vs group 2 (97.90 ± 26.00 vs 57.03 ± 14.99 pg/ml, p <0.01) and vs group 3 (39.93 ± 15.08, p <0.01). There was no statistical difference between groups 2 and 3. VEGF mRNA expression was similar among the groups.

Conclusions: Systemic bFGF increases smooth muscle content and VEGF protein in hypercholesterolemic rabbit corporeal tissue.

KEY WORDS: penis, rabbits, impotence, angiogenic growth factors, fibrosis, endothelium

Erectile dysfunction afflicts approximately 20 to 30 million men in the United States.[1] Normal penile erection is predominantly a vascular event that involves interaction between endothelial and smooth muscle cells in the corpus cavernosum.[1,2] The principle event in normal penile erection is trabecular smooth muscle relaxation mediated by the release of neurotransmitters from cavernous nerve terminals and nitric oxide from endothelial cells.[2,3] Although erectile dysfunction may result from psychological, neurological or arterial ischemic causes, the major etiology of erectile dysfunction is loss of vascular smooth muscle cell in the corpus cavernosum and a resulting decrease in vasomotor reactivity.[1]

Hypercholesterolemia is one of the major risk factors in the development of erectile dysfunction. In men each mmol/l increase in total cholesterol results in a 1.32-fold increase in the risk of erectile dysfunction.[4] In rabbits cholesterol feeding results in changes in corporeal tissue that depend on the amount of cholesterol in the diet and the duration of the diet. Kim et al administered a 1% cholesterol diet for 4 weeks and noted morphological changes in erectile tissue, including focal areas of endothelial dependent cell disruption, vacuolated endothelial dependent cells and an increase in lipid laden vesicles within smooth muscle cells.[5] Nehra et al reported

that 16 weeks of a 0.5% cholesterol diet significantly decreased corporeal smooth muscle cell content.[6] This decrease in corporeal vascular smooth muscle content is quantitatively and qualitatively similar to reductions seen in humans with erectile dysfunction.[7,8]

Cholesterol feeding is an established model of nontraumatic vascular injury and it is commonly used to study some aspects of atherosclerosis. In hypercholesterolemic animal models vascular injury begins with a breakdown of endothelial integrity without gross structural changes.[9] As the amount of endothelial injury increases, abnormalities become evident in the number and function of vascular smooth muscle cells that surround the endothelium.[5,9]

Angiogenesis is the growth and proliferation of blood vessels from existing vascular structures.[10] A number of angiogenic growth factors, including vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), are known to be present in vascular structures.[10–14] Proper expression of angiogenic growth factors is required for normal blood vessel growth during embryonic development, and angiogenic growth factors can act as survival factor for microvascular endothelium and help endothelial cells avoid cell death (apoptosis) when subject to injury.[10,13,14] This feature should be beneficial to corporeal endothelial cells during high cholesterol feeding. Prior series of our and other groups have demonstrated that VEGF administration has beneficial effects on smooth muscle cell content and function in the corporeal tissue of hypercholesterolemic rabbits.[14] FGF-2 or

bFGF is an 18 kDa protein with a strong affinity for heparin sulfate molecules on the cell surface and in the extracellular matrix.[12] Receptors for bFGF are found on multiple cell types and, therefore, bFGF has effects on the growth and proliferation of various cell types. In a preclinical study of hypercholesterolemic injury bFGF was able to protect arterial endothelium.[13] However, bFGF also has the potential to cause fibrosis.[15] Since little information is available on the potential use of bFGF in erectile dysfunction, we evaluated the effects of bFGF on erectile corporeal tissue in a hypercholesterolemic rabbit model of erectile dysfunction.

MATERIALS AND METHODS

Animal model. A total of 16 male New Zealand White rabbits weighing 2 to 3 kg were fed a 1% cholesterol diet (Harland Teklab, Madison, Wisconsin) for the whole duration of the study.[14] After 6 weeks the rabbits were randomly divided into 3 experimental groups. Group 1 (5 rabbits) received 2.5 μg recombinant bFGF in phosphate buffered saline solution (PBS) intravenously once and again 3 weeks later. Group 2 (6 rabbits) received 2.5 μg bFGF intravenously once and PBS 3 weeks later. Group 3 (5 rabbits) received PBS (placebo) intravenously each time. All rabbits were continuously fed the 1% cholesterol diet and were sacrificed 3 weeks after the second treatment. This regimen was chosen to be comparable to one used in humans with peripheral arterial disease.[16]

All protocols and procedures involving animals conformed to the Guidelines for Use of Laboratory Animals from the United States Department of Health and Human Services, and they were approved by the Duke University Animal Care and Use Committee. All animals received care in accordance with Principles of Laboratory Animal Care from the National Society for Medical Research and Guide for the Care and Use of Laboratory Animals from the National Institutes of Health (NIH), Publication No. 86–23, revised 1985.

Tissue procurement, histological section preparation, protein and mRNA isolation. At the end of the study all rabbits were deeply anesthetized with ketamine and xylazine. A blood sample was obtained for total serum cholesterol and penectomy was performed with careful dissection of the corpora cavernosa from the tunica albuginea before the rabbits were sacrificed.[14] The midpoint of the corporeal tissue was placed in 30% sucrose- PBS, pH 7.4, and mounted in cross section in optical cutting temperature. The remainder was snap frozen in liquid N_2. Cryostat sections (5 μm) were prepared on Superfrost Plus (Fisher Scientific, Pittsburgh, Pennsylvania) microscope slides for histological analysis. The remainder was snap frozen in liquid N_2 for protein extraction and RNA isolation.

For protein studies tissue samples were weighed, pulverized in liquid N_2 and homogenized in 3 to 5 × volumes 10 mM tris (hydroxymethyl) aminomethane (tris), pH 7.4, and 100 mM. NaCl using a Polytron (Brinkmann, Westbury, New York). The suspension was centrifuged twice at 8,000 × gravity at 4C for 15 minutes and the protein content of the supernatant was determined by Bradford assay. For RNA determination tissue samples were weighed and extracted with Trizol (Gibco BRL, Life Technologies, Inc., Grand Island, New York) total RNA isolation reagent according to manufacturer instructions with a bit modified method. Briefly, 20 to 50 mg tissue samples were homogenized in 200 μl Trizol, incubated for 10 minutes at room temperature and 40 μl chloroform were added. After centrifugation at 13,000 × gravity for 20 minutes at 4C the aqueous phase was collected and RNA was precipitated and washed by isopropyl alcohol and 75% ethanol, respectively. RNA concentration was determined by spectrophotometry.

Histological evaluation of smooth muscle, endothelial, cell and collagen content. To assess the content of vascular smooth muscle and endothelial cells in the corporeal tissue immunohistochemistry was performed using previously described antibodies and conditions.[14,17] Briefly, frozen sections were allowed to come to room temperature, and placed in ice-cold acetone for 10 minutes and then in PBS for 3, 5-minute washes. Blocking solution (10% normal horse serum in PBS) was applied for 20 minutes at room temperature. Mouse monoclonal antihuman smooth muscle actin antibody (Dako Corp., Carpinteria, California) (1:500) and mouse monoclonal antibody raised against the soluble extra-
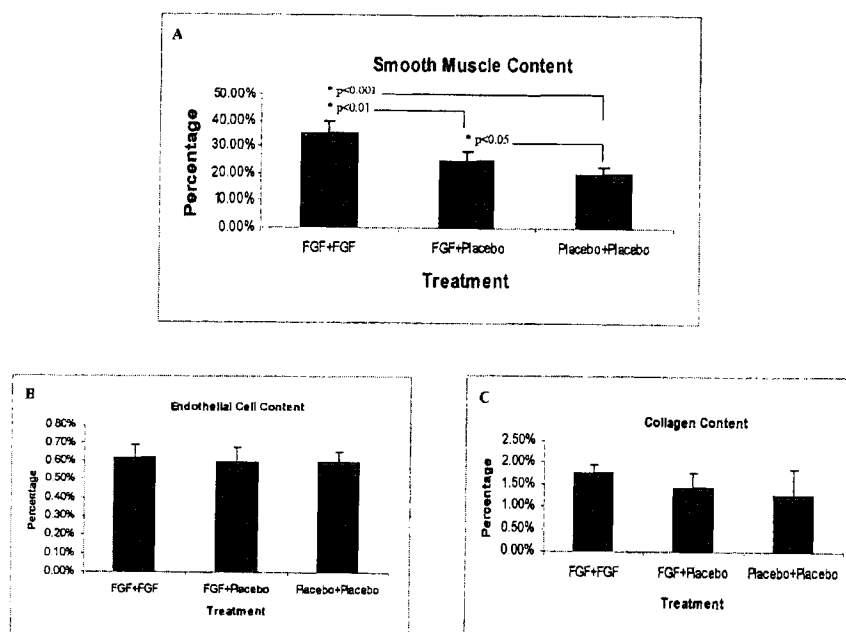
FIG. 1. Immunohistochemistry staining shows significantly increased corporeal smooth muscle content in group 1 with bFGF × 2 regimen than in group 2 with bFGF × 1 treatment (p <0.01) and group 3 with placebo (p <0.001). There was statistical difference between groups 2 and 3 (p <0.05) (A). Corporeal endothelial cell content was not changed among these 3 groups (B). On Masson trichrome staining corporeal collagen content was not significantly increased in group 1 and 2 compared with group 3 (p = 0.11 and 0.5, respectively) (C).

cellular domain of human Tie2 (a receptor tyrosine kinase expressed exclusively in endothelial cells) (Harlan, Indianapolis, Indiana) antibody (1:200) diluted in PBS was applied overnight at 4C. Sequential incubations with biotinylated antimouse IgG and avidin-biotin-peroxidase complex reagent using a Vectastain ABC Kit (Vector Laboratories, Burlingame, California) were performed according to manufacturer instructions. Levamisole was added to block endogenous alkaline phosphatase activity. Immune complexes were localized using alkaline phosphatase substrate Vector Red (Vector Laboratories). Sections were counterstained with hematoxylin, dehydrated and mounted with Permount (Fisher Scientific). The positive signal was red. Human vessels served as the positive control and PBS was used instead of the primary antibody as the negative control. Collagen content in corporeal tissue was measured by Sigma Accustain (Sigma Diagnostics, St. Louis, Missouri) Masson's trichrome stain according to manufacturer instructions. Collagen appears as a blue color.

For the content of smooth muscle cells, endothelial cells and collagen 6 randomly selected fields per sample at 200× magnification were acquired with a 1 × 70 microscope (Olympus Optical Corp., Melville, New York) and Premiere (Adobe, San Jose, California) software. Images were analyzed using NIH 1.62 image analysis software (NIH, Bethesda, Maryland). The overall smooth muscle (actin), endothelial cell (Tie2) or collagen (blue) area was quantified and the percent positive area per 1 certain field at 200× magnification was calculated. A signal reader blinded to sample type performed all analyses.

Measurement of bFGF protein, VEGF protein and mRNA. Protein concentrations of bFGF and VEGF in corporeal tissue protein lysates were determined using a solid-state enzyme-linked immunoassay (ELISA) system with a Quantikine bFGF and VEGF ELISA Kit (R & D Systems, Minneapolis, Minnesota), as previously described.[17] Assay sensitivity was 5 and 10 pg/ml for recombinant VEGF and bFGF, respectively. Results are expressed in pg/ml based on standard recombinant protein curves.[17] The assay was validated for up to 100 μg total protein per well. Semiquantitative reverse transcriptase (RT)-polymerase chain reaction (PCR) was performed using 1 μg total RNA for first strand cDNA synthesis. RNA was reverse transcribed for 12 minutes at 42C using Multi-Scribe (AB Applied Biosystems, Foster City, California) RT and random hexamer primers. The cDNA products were amplified by PCR using AmpliTaq Gold DNA Polymerase with a GeneAmp Gold RNA PCR Reagent Kit (AB Applied Biosystems, Foster City, California) according to manufacturer instructions. Primer sequences used for rabbit VEGF-A were 5'-TACTGCCAGCC-GATCGAGACC-3' (sense) and 5'-CTTTGGTCTGCATTCA-CATTTG-3' (antisense). As a control, cDNA for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was amplified. PCR products were analyzed by 1.5% agarose gel electrophoresis.

Statistical analysis. Unless otherwise stated results are expressed as the mean ± SD. Statistical significance was evaluated by Student's t test for paired or unpaired variables with p <0.05 considered statistically significant.

RESULTS

Total serum cholesterol values were markedly elevated in all 3 groups. Final mean serum cholesterol levels in groups 1 to 3 were 1,368.8 ± 211.7, 1,370.3 ± 627.6 and 1,249.5 ± 69.8 mg/dl, respectively.

Systemic administration of bFGF led to differences in smooth muscle cell content in corporeal tissue (fig. 1, A). In group 1 the fraction of smooth muscles in erectile corporeal tissue was 35.24% ± 4.25%, which was significantly higher than in group 2 (24.79% ± 3.39%, p <0.01) and group 3 (19.68% ± 2.94%, p <0.001). The difference between groups 2 and 3 was also statistically significant (p <0.05). Figure 2, A and B shows a representative example. The content of endothelial cells in corporeal tissue was not significantly different among groups 1 to 3 (0.62% ± 0.07%, 0.60% ± 0.08% and 0.60% ± 0.08%, respectively, fig. 1, B). The fraction of collagen content in corporeal tissue was not significantly different among groups 1 to 3 (1.76% ± 0.20%, 1.44 ± 0.32% and 1.25% ± 0.60%, respectively, fig. 1, C). Figure 2, C and D shows representative collagen staining in groups 1 and 3.

Corporeal tissue levels of bFGF protein were 314 ± 98, 393 ± 63 and 314 ± 64 pg/ml soluble protein in groups 1 to 3, respectively. On ELISA the amount of VEGF protein in corporeal tissue detected was higher in group 1 than in groups
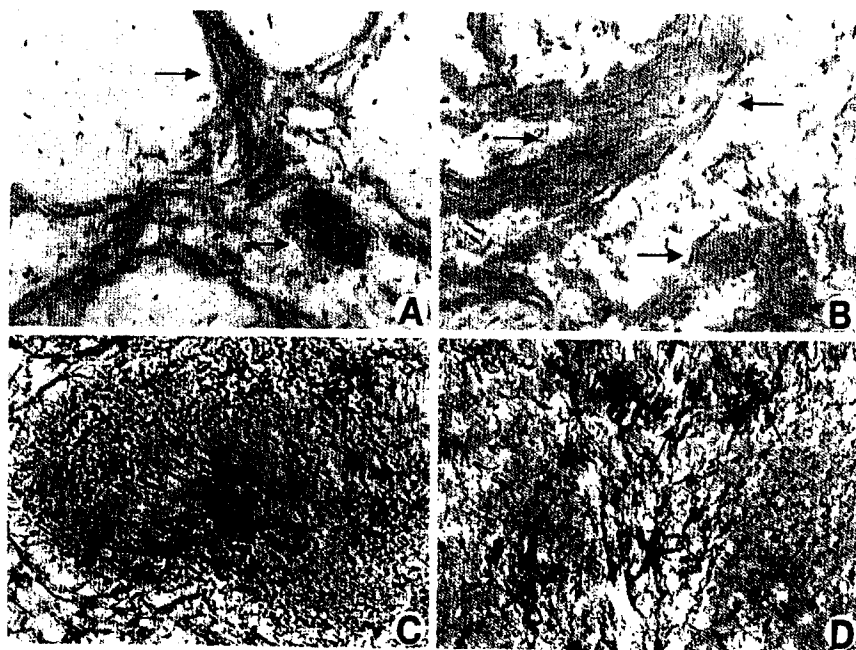

FIG. 2. Representative corporeal smooth muscle content measured by immunohistochemistry in groups 3 (*A*) and 1 (*B*). Smooth muscle actin antibody, reduced from ×200. Representative corporeal collagen content in groups 3 (*C*) and 1 (*D*). Masson's trichrome stain, reduced from ×200.

2 and 3 (97.90 ± 26.00 vs 57.03 ± 14.99 and.93 ± 15.08 pg/ml, each vs group 1 p <0.01, fig. 3, A). The difference between groups 2 and 3 was not statistically significant (p = 0.08). By RT-PCR there was no difference in VEGF-A gene expression in corporeal tissue among these 3 groups (fig. 3, B).

DISCUSSION

Erectile dysfunction is a significant health problem that is also a frequent manifestation of atherosclerotic vascular disease that leads to abnormal interaction between endothelial cells and smooth muscle cells in the corpus cavernosum.[1] Hypercholesterolemia induces injury to endothelial and vascular smooth muscle cells, resulting in abnormal function and content in preclinical and human corporeal tissues.[1,2,5,6] Angiogenesis is the growth and proliferation of blood vessels from existing vascular structures and, in addition to promoting endothelial proliferation, angiogenic growth factors like VEGF and bFGF have the ability to modulate favorably vascular injury.[10] Prior studies from our and other groups have shown that VEGF administration can improve corporeal smooth muscle function in vitro and ex vivo in models of erectile dysfunction.[14,18] In models of arterial injury bFGF has been shown to limit the extent of injury, although bFGF is also capable of stimulating smooth cells to secrete collagen and cause fibrosis in target tissue.[12,13,15] Since little information is available on the effects of bFGF on corporeal tissue histology in a preclinical model of erectile dysfunction, we

administered intravenous bFGF, mimicking a regimen that has been efficacious in humans with peripheral arterial disease.[16] The major findings of the current study were that the systemic administration of bFGF results in an increase in vascular smooth muscle with no change in endothelial cell content, an increase in VEGF protein expression and no significant increase in corporeal tissue fibrosis. Trabecular smooth muscle content is the key structure in normal erectile function and the degree of loss of corporeal smooth muscle content correlates with the extent of impairment in corporeal veno-occlusive function. Therefore, our findings suggest that bFGF may be valuable in the therapeutic modulation of erectile dysfunction.

Hypercholesterolemia causes a nontraumatic form of vascular injury that initially targets the endothelium but ultimately leads to injury to and loss of vascular smooth muscle cells.[9] In vitro experiments have shown that angiogenic growth factors such as bFGF and VEGF are not only capable of stimulating endothelial cells proliferation, but also serve as an endothelial cells survivor factors to protect them from injury.[10] VEGF has been shown to be effective in preclinical models of erectile dysfunction[14,18] and the current study extends these observations to another angiogenic growth factor, bFGF. Te et al reported that bFGF is present at high levels in rat corporeal tissue.[11] In the same study they noted that nerve growth proliferating activity in penile tissue was inhibited by FGF blocking antibodies.[11] For VEGF up-regulation of endothelial nitric oxide synthase may have a role in the beneficial effects that follow VEGF treatment.[19] Additional experiments are needed to determine whether VEGF and bFGF act through similar mechanisms to exert beneficial effects on corporeal tissue.

In our study intravenous bFGF treatment significantly increased (approximately 2.5-fold) VEGF-A protein expression in corporeal tissue 3 weeks after the last treatment. VEGF-A gene expression did not appear different among the groups and this finding would suggest that VEGF regulation in corporeal tissue occurs at the post-transcriptional level. Cells responsible for the change in VEGF are unknown but VEGF may be produced by endothelial, vascular smooth or other cells present in corporeal tissue.[14,18] Burchardt et al detected the expression of VEGF splice variants in adult rat and human penile tissues using RT-PCR.[20] The primers used in our study detect the smallest and, therefore, all VEGF splice variants. The antibody used in ELISA detects VEGF isoforms of 121 to 189 amino acids (product information data).[17] Additional studies are needed to determine any differential effects on the isoforms and whether the increase in VEGF protein was the result of increased smooth muscle cell content.

Although it is still investigational, therapeutic angiogenesis is an exciting and promising treatment strategy for peripheral artery disease and ischemic heart disease. Our current study added to other studies of VEGF administration expands the spectrum of the potential use of angiogenic growth factors to modulate forms of vascular injury, including the treatment of erectile dysfunction. Additional studies are needed to determine the physiological correlates of the favorable histological and biochemical findings reported in this study.
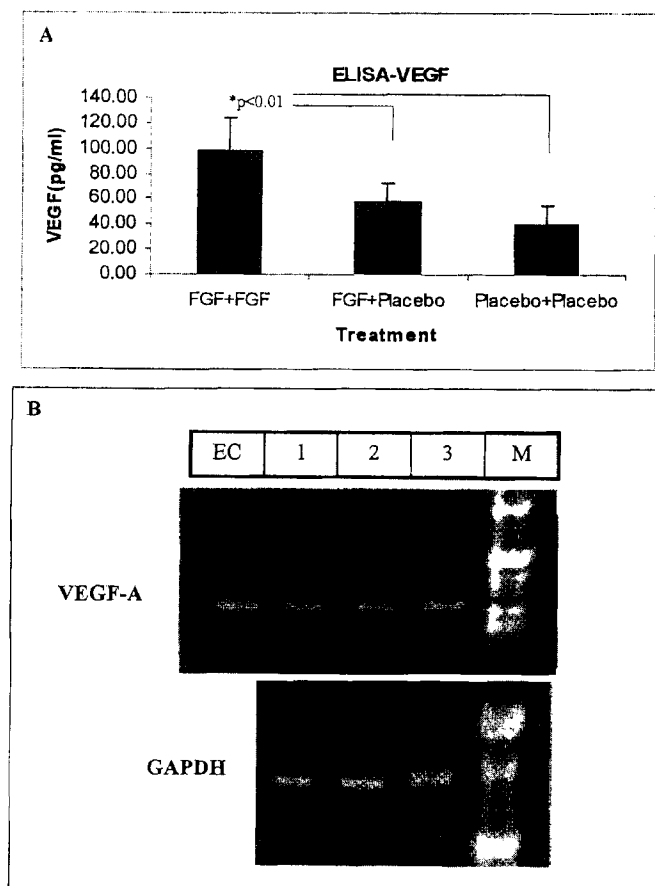
FIG. 3. On ELISA VEGF protein in corporeal tissue was higher in group 1 than in group 2 or 3 (each p <0.01). There was no statistical difference between groups 2 and 3 (p = 0.08) (A). On RT-PCR there was no difference in rabbit VEGF-A mRNA expression relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in corporeal tissue among groups 1 to 3 (lanes 1 to 3, respectively) (B). Lane EC, endothelial cell mRNA as positive control. M, 1 kb DNA ladder.

REFERENCES

1. Lue, T. F.: Erectile dysfunction. N Engl J Med, 342: 1802, 2000
2. Fournier, G. R., Jr., Juenemann, K.-P., Lue, T. F. and Tanagho, E. A.: Mechanisms of venous occlusion during canine penile erection: an anatomic demonstration. J Urol, 137: 163, 1987

3. Burnett, A. L., Lowenstein, C. J., Bredt, D. S., Chang, T. S. and Snyder, S. H.: Nitric oxide: a physiologic mediator of penile erection. Science, **257:** 401, 1992
4. Wei, M., Macera, C. A., Davis, D. R., Hornung, C. A., Nankin, H. R. and Blair, S. N.: Total cholesterol and high density lipoprotein cholesterol as important predictors of erectile dysfunction. Am J Epidemiol, **140:** 930, 1994
5. Kim, J. H., Klyachkin, M. L., Svendsen, E., Davies, M. G., Hagen, P.-O. and Carson, C. C., III: Experimental hypercholesterolemia in rabbits induces cavernosal atherosclerosis with endothelial and smooth muscle cell dysfunction. J Urol, **151:** 198, 1994
6. Nehra, A., Azadzoi, K. M., Moreland, R. B., Pabby, A., Siroky, M. B., Krane, R. J. et al: Cavernosal expandability is an erectile tissue mechanical property which predicts trabecular histology in an animal model of vasculogenic erectile dysfunction. J Urol, **159:** 2229, 1998
7. Wespes, E., Sattar, A. A., Golzarian, J., Wery, D., Daoud, N. and Schulman, C. C.: Corporeal veno-occlusive dysfunction: predominantly intracavernous muscular pathology. J Urol, **157:** 1678, 1997
8. Sattar, A. A., Merckx, L. A. and Wespes, E.: Penile electromyography and its smooth muscle content: interpretation of 25 impotent patients. J Urol, **155:** 909, 1996
9. Azadzoi, K. M. and Goldstein, I.: Erectile dysfunction due to atherosclerotic vascular disease: the development of an animal model. J Urol, **147:** 1675, 1992
10. Folkman, J.: Seminars in Medicine of the Beth Israel Hospital, Boston. Clinical applications of research on angiogenesis. N Engl J Med, **333:** 1757, 1995
11. Te, A. E., Santarosa, R. P., Koo, H. P., Buttyan, R., Greene, L. A., Kaplan, S. A. et al: Neurotrophic factors in the rat penis. J Urol, **152:** 2167, 1994
12. Nugent, M. A. and Iozzo, R. V.: Fibroblast growth factor-2. Int J Biochem Cell Biol, **32:** 115, 2000
13. Meurice, T., Bauters, C., Vallet, B., Corseaux, D., van Belle, E., Hamon, M. et al: bFGF restores endothelium-dependent responses of hypercholesterolemic rabbit thoracic aorta. Am J Physiol, **272:** H613, 1997
14. Byrne, R. R., Henry, G. D., Rao, D. S., Huynh, T. T. T., Pippen, A. M., Annex, B. H. et al: Vascular endothelial growth factor restores corporeal smooth muscle function in vitro. J Urol, **165:** 1310, 2001
15. Inoue, Y., King, T. E., Jr., Barker, E., Daniloff, E. and Newman, L. S.: Basic fibroblast growth factor and its receptors in idiopathic pulmonary fibrosis and lymphangioleiomyomatosis. Am J Respir Crit Care Med, **166:** 765, 2002
16. Lederman, R. J., Mendelsohn, F. O. Anderson, R. D., Saucedo, J. F., Tenaglia, A. N., Hermiller, J. B. et al: Therapeutic angiogenesis with recombinant fibroblast growth factor-2 for intermittent claudication (the TRAFFIC study): a randomised trial. Lancet, **359:** 2053, 2002
17. Cherwek, D. H., Hopkins, M. B., Thompson, M. J., Annex, B. H. and Taylor, D. A.: Fiber type-specific differential expression of angiogenic factors in response to chronic hindlimb ischemia. Am J Physiol Heart Circ Physiol, **279:** H932, 2000
18. Lee, M.-C., El-Sakka, A. I., Graziottin, T. M., Ho, H.-C., Lin, C.-S. and Lue, T. F.: The effect of vascular endothelial growth factor on a rat model of traumatic arteriogenic erectile dysfunction. J Urol, **167:** 761, 2002
19. Lin, C. S., Ho, H. C., Chen, K. C., Lin, G., Nunes, L. and Lue, T. F.: Intracavernosal injection of vascular endothelial growth factor induces nitric oxide synthase isoforms. BJU Int, **89:** 955, 2002
20. Burchardt, T., Burchardt, M., Chen, M. W., Buttyan, R., de la Taille, A., Shabsigh, A. et al: Expression of VEGF splice variants 144/145 and 205/206 in adult male tissues. IUBMB Life, **48:** 405, 1999